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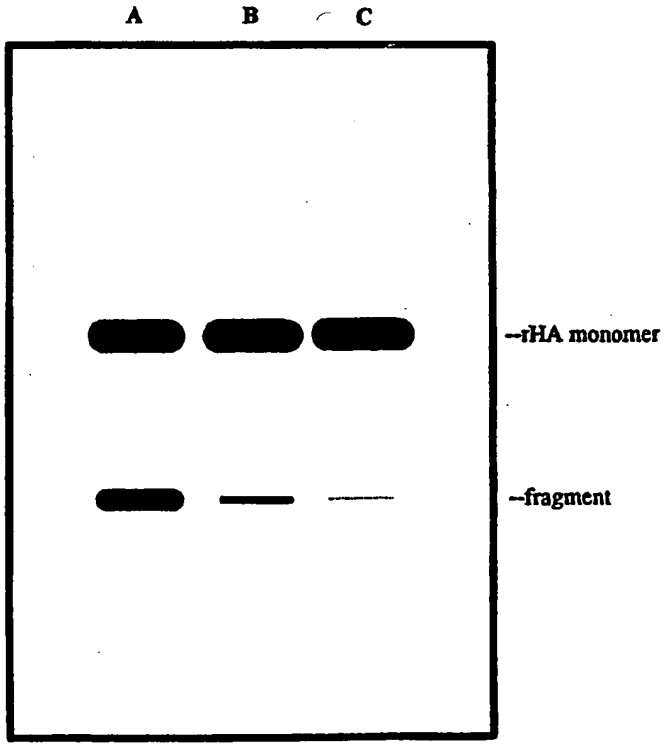
(54) Title: YEAST STRAINS AND MODIFIED ALBUMINS

(57) Abstract

Albumin, for example human albumin, is expressed and secreted in yeast which has been mutated to lack the yeast aspartyl protease 3 (Yap3p) or its equivalent, thereby reducing the production of a 45kD albumin fragment. A further reduction is achieved by additionally deleting the Kex2p function. Alternatively, a modified albumin is prepared which is not susceptible to Yap3p cleavage, for example human albumin which is R410A, K413Q and K414Q.



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YEAST STRAINS AND MODIFIED ALBUMINS

Field of the invention

The present invention relates to the production of recombinant human albumin (rHA) by yeast species.

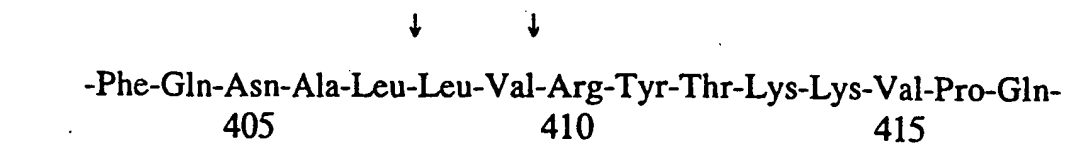
Background and prior art

Human serum albumin (HSA) is a protein of 585 amino acids that is responsible for a significant proportion of the osmotic pressure of serum, and also functions as a carrier of endogenous and exogenous ligands. It is used clinically in the treatment of patients with severe burns, shock, or blood loss, and at present is produced commercially by extraction from human blood. The production of recombinant human albumin (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

In recent years yeast species have been widely used as a host organisms for the production of heterologous proteins (reviewed by Romanos *et al*, 1992), including rHA (Sleep *et al*, 1990, 1991; Fleer *et al*, 1991). Yeasts are readily amenable to genetic manipulation, can be grown to high cell density on simple media, and as eukaryotes are suitable for production of secreted as well as cytosolic proteins.

When *S. cerevisiae* is utilised to produce rHA, the major secreted protein is mature 67kDa albumin. However, a 45kDa N-terminal fragment of rHA is also observed (Sleep *et al*, 1990). A similar fragment is obtained when rHA is expressed in *Kluyveromyces* sp. (Fleer *et al*, 1991) and *Pichia pastoris* (EP 510 693). The fragment has the same N-terminal amino acid sequence as mature rHA, but the carboxy terminus is heterogeneous and occurs between

Phe[403] and Val[409] with the most common termini being Leu[407] and Val[409] (Geisow *et al*, 1991), as shown below.



The amount of fragment produced, as a percentage of total rHA secreted, varies with both the strain and the secretion leader sequence utilised, but is never reduced to zero (Sleep *et al*, 1990). We have also found that the amount of fragment produced in high cell density fermentation (75-100g/L cell dry weight) is approximately five times higher than in shake flask cultures.

The 45kDa albumin fragment is not observed in serum-derived human serum albumin (HSA), and its presence as non-nature-identical material in the recombinant product is undesirable. The problem addressed by the present invention is to reduce the amount of the 45kDa fragment in the product. The simplest and most obvious approach would have been to have purified it away from the full length albumin, as proposed by Gist-brocades in EP 524 681 (see especially page 4, lines 17-22). However, we have chosen a different approach, namely to try to avoid its production in the first place.

Sleep *et al* (1990) postulated that rHA fragment is produced within the cell and is not the result of extra-cellular proteolysis. These authors codon-optimised the HSA cDNA from Glu[382] to Ser[419] but this had no effect on production of rHA fragment. They noted that a potential Kex2p processing site in the rHA amino acid sequence, Lys[413]Lys[414], is in close proximity to the heterogeneous carboxy terminus of the fragment, but neither use of a *kex2* host strain (ie a strain harbouring a mutation in the *KEX2* gene such that it does not produce the Kex2p protease), nor removal of the potential cleavage site by site-directed

mutagenesis of the codon for Lys^{414}, resulted in reduction in the amount of the fragment.

There is a vast array of yeast proteases which could, in principle, be degrading a desired protein product, including (in *S. cerevisiae*) yscA, yscB, yscY, yscS, other vacuolar proteinases, yscD, yscE, yscF (equivalent to kex2p), yscα, yscIV, yscG, yscH, yscJ, yscE and kex1.

Bourbonnais *et al* (1991) described an *S. cerevisiae* endoprotease activity specific for monobasic sites, an example of which (Arg^{410}) exists in this region of albumin. This activity was later found to be attributable to yeast aspartyl protease 3 (Yap3) (Bourbonnais *et al*, 1993), an enzyme which was originally described by Egel-Mitani *et al* (1990) as an endoprotease similar to Kex2p in specificity, in that it cleaved at paired basic residues. Further work suggested that Yap3p is able to cleave monobasic sites and between, and C-terminal to, pairs of basic residues, but that cleavage at both types of sites is dependent on the sequence context (Azaryan *et al*, 1993; Cawley *et al*, 1993).

As already discussed, the region of the C-terminus of rHA fragment contains both a monobasic (Arg^{410}) and a dibasic site ($Lys^{413}Lys^{414}$). However, even though a Kex2p-like proteolytic activity is present in human cells and is responsible for cleavage of the pro sequence of HSA C-terminal to a pair of arginine residues, the fragment discussed above is not known to be produced in humans. This indicates that the basic residues Arg^{410}, Lys^{413} and Lys^{414} are not recognised by this Kex2p-like protease, in turn suggesting that this region of the molecule may not be accessible to proteases in the secretory pathway. Thus, the Yap3p protease could not have been predicted to be responsible for the production of the 45kDa fragment. In addition, Egel-Mitani *et al* (1990 *Yeast* **6**, 127-137) had shown Yap3p to be similar to Kex2p in cleaving the MFα propheromone. Since removal of the Kex2p function alone does not

reduce the amount of the fragment produced, there was no reason to suppose that removal of the Yap3p function would be beneficial. Indeed, Bourbonnais *et al* (1993) showed that *yap3* strains had a decreased ability to process pro-somatostatin, and therefore taught away from using *yap3* strains in the production of heterologous proteins.

Summary of the invention

The solution to the problem identified above is, in accordance with the invention, to avoid or at least reduce production of the fragment in the initial fermentation, rather than to remove it during purification of the albumin. We have now found that, out of the 20 or more yeast proteases which are so far known to exist, it is in fact the Yap3p protease which is largely responsible for the 45kD fragment of rHA produced in yeast. The present invention provides a method for substantially reducing the amount of a 45kDa fragment produced when rHA is secreted from yeast species. The reduction in the amount of fragment both improves recovery of rHA during the purification process, and provides a higher quality of final product. A further, and completely unexpected, benefit of using *yap3* strains of yeast is that they can produce 30-50% more rHA than strains having the Yap3p function. This benefit cannot be accounted for merely by the reduction of rHA fragment from ~15% to 3-5%.

Thus, one aspect of the present invention provides a process for preparing albumin by secretion from a yeast genetically modified to produce and secrete the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.

Preferably, the said proteolytic activity is an endoprotease activity specific for monobasic sites and for paired basic amino acids in a polypeptide.

Suitably, the yeast is *S. cerevisiae* which lacks a functional *YAP3* gene. However, the invention is not limited to the use of *S. cerevisiae*, since the problem of 45 kDa fragment production is found also in other yeast genera, for example *Pichia* and *Kluyveromyces*, which shows that they have equivalent proteases (ie Yap3p proteolytic activity); see Clerc *et al* (1994), page 253. We have confirmed this by hybridisation analysis to locate homologues of Yap3p in non-*Saccharomyces* genera. A gene is regarded as a homologue, in general, if the sequence of the translation product has greater than 50% sequence identity to Yap3p. In non-*Saccharomyces* genera, the Yap3p-like protease and its gene may be named differently, but this does not of course alter their essential nature.

The level of fragment can be reduced still further if, as well as substantially eliminating the Yap3p proteolytic activity, the Kex2p function is also substantially eliminated even though, as mentioned above, elimination of the Kex2p function alone does not affect the level of fragment. As in the case of Yap3p, the Kex2p function is not restricted to *Saccharomyces*; see Gellissen *et al* (1992), especially the sentence bridging pages 415 and 416, showing that *Pichia* has a Kex2p function. The genes encoding the Kex2p equivalent activity in *Kluyveromyces lactis* and *Yarrowia lipolytica* have been cloned (Tanguy-Rougeau *et al*, 1988; Enderlin & Ogrydziak, 1994).

A suitable means of eliminating the activity of a protease is to disrupt the host gene encoding the protease, thereby generating a non-reverting strain missing all or part of the gene for the protease (Rothstein, 1983). Alternatively, the activity can be reduced or eliminated by classical mutagenesis procedures or by the introduction of specific point mutations by the process of transplacement (Winston *et al*, 1983). Preferably, the activity of the enzyme is reduced to at most 50% of the wild-type level, more preferably no more than 25%, 10% or 5%, and most preferably is undetectable. The level of Yap3p proteolytic

activity may be measured by determining the production of the 45 kDa fragment, or by the ^{125}I-β_h-lipoprotein assay of Azaryan *et al* (1993), also used by Cawley *et al* (1993). Kex2p proteolytic activity may similarly be measured by known assays, for example as set out in Fuller *et al* (1989).

The albumin may be a human albumin, or a variant thereof, or albumin from any other animal.

By "variants" we include insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or non-immunogenic properties of albumin. In particular, we include naturally-occurring polymorphic variants of human albumin; fragments of human albumin which include the region cleaved by Yap3p, for example those fragments disclosed in EP 322 094 (namely HSA (1-n), where n is 369 to 419) which are sufficiently long to include the Yap3p-cleaved region (ie where n is 403 to 419); and fusions of albumin (or Yap3p-cleavable portions thereof) with other proteins, for example the kind disclosed in WO 90/13653.

By "conservative substitutions" is intended swaps within groups such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

Such variants may be made using the methods of protein engineering and site-directed mutagenesis as described below.

A second aspect of the invention provides a modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with the *S. cerevisiae* yeast aspartyl protease 3 (Yap3p) when expressed in yeast, characterised in that the modified albumin is not susceptible to such cleavage.

Preferably, the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein. Suitably, the said monobasic amino acid is arginine. Conveniently, the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin, especially any of Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg. Thus, in one particular embodiment, the naturally-occurring albumin is human albumin and the modified protein lacks Arg^{410} and, optionally, one or both $Lys^{413}Lys^{414}$ lysines. For example, the modified albumin may be human albumin having the amino acid changes R410A, K413Q, K414Q. Equivalent modifications in bovine serum albumin include replacing the Arg^{408} and/or one or both of $Arg^{411}Lys^{412}$. The person skilled in the art will be able to identify monobasic sites and pairs of basic residues in other albumins without difficulty.

The numbering of the residues corresponds to the sequence of normal mature human albumin. If the albumin is a variant (for example a polymorphic form) having a net deletion or addition of residues N-terminal to the position identified, then the numbering refers to the residues of the variant albumin which are aligned with the numbered positions of normal albumin when the two sequences are so aligned as to maximise the apparent homology.

A third aspect of the invention provides a polynucleotide encoding such a modified albumin.

The DNA is expressed in a suitable yeast (either the DNA being for a modified albumin, or the yeast lacking the Yap3p function) to produce an albumin. Thus, the DNA encoding the albumin may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate yeast cell for the expression and production of the albumin.

The DNA encoding the albumin may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The vector is then introduced into the host through standard techniques and, generally, it will be necessary to select for transformed host cells.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression and secretion of the albumin, which can then be recovered, as is known.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps). Other yeast expression plasmids are disclosed in EP-A-258 067, EP-A-286 424 and EP-A-424 117.

The polynucleotide coding sequences encoding the modified albumin of the invention may have additional differences to those required to produce the modified albumin. For example, different codons can be substituted which code for the same amino acid(s) as the original codons. Alternatively, the substitute codons may code for a different amino acid that will not affect the activity or immunogenicity of the albumin or which may improve its activity

or immunogenicity, as well as reducing its susceptibility to a Yap3p protease activity. For example, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle (1985). Since such modified coding sequences can be obtained by the application of known techniques to the teachings contained herein, such modified coding sequences are within the scope of the claimed invention.

Exemplary genera of yeast contemplated to be useful in the practice of the present invention are *Pichia, Saccharomyces, Kluyveromyces, Candida, Torulopsis, Hansenula* (now reclassified as *Pichia*), *Histoplasma, Schizosaccharomyces, Citeromyces, Pachysolen, Debaromyces, Metschunikowia, Rhodosporidium, Leucosporidium, Botryoascus, Sporidiobolus, Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of *Pichia, Saccharomyces, Kluyveromyces, Yarrowia* and *Hansenula*. Examples of *Saccharomyces sp.* are *S. cerevisiae, S. italicus* and *S. rouxii*. Examples of *Kluyveromyces sp.* are *K. fragilis* and *K. lactis*. Examples of *Hansenula (Pichia) sp.* are *H. polymorpha* (now *Pichia angusta*), *H. anomala* (now *P. anomala*) and *P. pastoris*. *Y. lipolytica* is an example of a suitable *Yarrowia* species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference. Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α-mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GPD1* promoter, and hybrid promoters involving hybrids of parts of 5′ regulatory regions with parts of 5′

regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

Convenient regulatable promoters for use in *Schizosaccharomyces pombe* are the thiamine-repressible promoter from the *nmt* gene as described by Maundrell (1990) and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990).

Methods of transforming *Pichia* for expression of foreign genes are taught in, for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857 467, incorporated herein by reference), and *Pichia* expression kits are commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen Corp., San Diego, California. Suitable promoters include *AOX1* and *AOX2*.

The Gellissen *et al* (1992) paper mentioned above and Gleeson *et al* (1986) *J. Gen. Microbiol.* **132**, 3459-3465 include information on *Hansenula* vectors and transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991, Fleer *et al* (1991) and other publications from Rhône-Poulenc Rorer teach how to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being *PGK1*.

The transcription termination signal is preferably the 3′ flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3′ flanking sequences may, for example, be those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

The albumin is initially expressed with a secretion leader sequence, which may be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae*

include that from the mating factor α polypeptide (MFα-1) and the hybrid leaders of EP-A-387 319. Such leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. When the yeast strain lacks Kex2p activity (or equivalent) as well as being *yap3*, it may be advantageous to choose a secretion leader which need not be cleaved from the albumin by Kex2p. Such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-sequence of MFα-1, β-glucanase (*BGL2*) and killer toxin; *S. diastaticus* glucoamylase II; *S. carlsbergensis* α-galactosidase (*MEL1*); *K. lactis* killer toxin; and *Candida* glucoamylase.

Various non-limiting embodiments of the invention will now be described by way of example and with reference to the accompanying drawings in which:

Figure 1 is a general scheme for the construction of mutated rHA expression plasmids, in which HA is a human albumin coding sequence, L is a sequence encoding a secretion leader, P is the PRB1 promoter, T is the ADH1 terminator, amp is an ampicillin resistance gene and LEU2 is the leucine selectable marker;

Figure 2 is a drawing representing a Western blot analysis of mutant rHA secreted by *S. cerevisiae*, in which Track A represents the culture supernatant from DB1 cir° pAYE316 (normal rHA), Track B represents the culture supernatant from DB1 cir$^+$ pAYE464 (alteration 1), and Track C represents the culture supernatant from DB1 cir$^+$ pAYE468 (alteration 3);

Figure 3 is a scheme of the construction of pAYE515;

Figure 4 is a comparison of rHA fragment production by wild-type and protease-disrupted strains, presented as a drawing of an anti-HSA Western blot

of culture supernatant from shake flask cultures separated by non-reducing 10% SDS/PAGE, in which Track A corresponds to DB1 cir° pAYE316, Track B corresponds to DXY10 cir° pAYE316 (*yap3* strain), and Track C corresponds to ABB50 cir° pAYE316 (*yap3*, *kex2* strain);

Figure 5 is similar to Figure 4 but shows Coomassie Brilliant Blue stained 12.5% SDS Phastgel (Pharmacia) of culture supernatants from fed batch fermentations, namely Track D for the HSA standard, Track E for DB1 cir° pAYE316, Track F for DB1 Δ*kex2* cir° pAYE522, and Track G for DXY10 cir° pAYE522; and

Figure 6 is a scheme for the construction of pAYE519.

Detailed description of the invention

All standard recombinant DNA procedures are as described in Sambrook *et al* (1989) unless otherwise stated. The DNA sequences encoding HSA are derived from the cDNA disclosed in EP 201 239.

Example 1: Modification of the HSA cDNA.

In order to investigate the role of endoproteases in the generation of rHA fragment, the HSA cDNA (SEQ1 (which includes a sequence encoding the artificial secretion leader sequence of WO 90/01063)) was modified by site-directed mutagenesis. Three separate changes were made to the HSA sequence (SEQ2). The first, using the mutagenic primer FOG1, changed the Arg^{410} codon only, replacing it with an Ala codon, leaving intact the dibasic site, $Lys^{413}Lys^{414}$. The second change, using primer FOG2, changed the residues 407-409, including the C-terminal residues of fragment, from LeuLeuVal to AlaValAla. The third change, using the primer FOG3, altered residues 410-

414 from **ArgTyrThrLysLys** (SEQ3) to **AlaTyrThrGlnGln** (SEQ4). The oligonucleotides encoded not only the amino acid changes, but also conservative base changes that create either a *Pvu*II or an *Spe*I restriction site in the mutants to facilitate detection of the changed sequences.

Single-stranded DNA of an M13mp19 clone, mp19.7 (EP 201 239; Figure 2), containing the HSA cDNA was used as the template for the mutagenesis reactions using the *In Vitro* Mutagenesis System, Version 2 (Amersham International plc) according to the manufacturer's instructions. Individual plaques were selected and sequenced to confirm the presence of the mutations. Double stranded RF DNA was then made from clones with the expected changes and the DNA bearing the mutation was excised on an *Xba*I/*Sac*I fragment (Figure 1). This was used to replace the corresponding wild-type fragment of pAYE309 (EP 431 880; Figure 2). The presence of the mutated *Xba*I/*Sac*I fragment within the plasmid was checked by digesting with *Pvu*II or *Spe*I as appropriate. These *Hin*dIII fragments were excised and inserted into the expression vector pAYE219 (Figure 1) to generate the plasmids pAYE464 (alteration 1, R410A), pAYE470 (alteration 2, L407A, L408V, V409A) and pAYE468 (alteration 3, R410A, K413Q, K414Q). These expression plasmids comprise the *S. cerevisiae PRB1* promoter (WO 91/02057) driving expression of the HSA/MFα1 leader sequence (WO 90/01063) fused in-frame with the mutated HA coding sequence which is followed by the *ADH1* transcription terminator. The plasmids also contain part of the 2μm plasmid to provide replication functions and the *LEU2* gene for selection of transformants.

pAYE464, pAYE470 and pAYE468 were introduced into *S. cerevisiae* DB1 cir$^+$ (*a*, *leu2*; Sleep *et al*, 1990) by transformation and individual transformants were grown for 3 days at 30°C in 10ml YEPS (1% ^{w}/$_v$ yeast extract, 2% ^{w}/$_v$ peptone, 2% ^{w}/$_v$ sucrose) and then the supernatants were examined by anti-HSA Western blot for the presence of the rHA fragment. The Western blots clearly

showed that fragment was still produced by the strains harbouring pAYE464, although the level was reduced slightly compared to the control expressing wild-type rHA. The mutations in the plasmid pAYE470 appeared to have no effect on the generation of fragment. However, DB1 cir$^+$ pAYE468 showed a novel pattern of HSA-related bands, with little or no fragment.

One example of each of DB1 cir$^+$ pAYE464 and DB1 cir$^+$ pAYE468 were grown to high cell density by fed batch culture in minimal medium in a fermenter (Collins, 1990). Briefly, a fermenter of 10L working volume was filled to 5L with an initial batch medium containing 50 mL/L of a concentrated salts mixture (Table 1), 10 mL/L of a trace elements solution (Table 2), 50 mL/L of a vitamins mixture (Table 3) and 20 g/L sucrose. An equal volume of feed medium containing 100 mL/L of the salts mixture, 20 mL/L of the trace elements mixture, 100 mL/L of vitamins solution and 500 g/L sucrose was held in a separate reservoir connected to the fermenter by a metering pump. The pH was maintained at 5.7 $\pm$ 0.2 by the automatic addition of ammonium hydroxide or sulphuric acid, and the temperature was maintained at 30°C. The stirrer speed was adjusted to give a dissolved oxygen tension of >20% air saturation at 1 v/v/min air flow rate.

Table 1. Salts Mixture

Chemical	Concentration (g/L)
KH_2PO_4	114.0
$MgSO_4$	12.0
$CaCl_2.6H_2O$	3.0
Na_2EDTA	2.0

Table 2. Trace Elements Solution

Chemical	Concentration (g/L)
$ZnSO_4.7H_2O$	3.0
$FeSO_4.7H_2O$	10.0
$MnSO_4.4H_2O$	3.2
$CuSO_4.5H_2O$	0.079
H_3BO_3	1.5
KI	0.2
$Na_2MoO_4.2H_2O$	0.5
$CoCl_2.6H_2O$	0.56
H_3PO_4	75mL/L

Table 3. Vitamins Solution

Chemical	Concentration (g/L)
Ca pantothenate	1.6
Nicotinic acid	1.2
m inositol	12.8
Thiamine HCl	0.32
Pyridoxine HCl	0.8
Biotin	0.008

The fermenter was inoculated with 100 mL of an overnight culture of *S. cerevisiae* grown in buffered minimal medium (Yeast nitrogen base [without amino acids, without ammonium sulphate, Difco] 1.7 g/L, $(NH_4)_2SO_4$ 5 g/L, citric acid monohydrate 6.09 g/L, Na_2HPO_4 20.16 g/L, sucrose 20 g/L, pH6.5). The initial batch fermentation proceeded until the carbon source had been consumed, at which point the metering pump was switched on and the addition of feed was computer controlled (the micro MFCS system, B. Braun,

Melsungen, Germany) using an algorithm based on that developed by Wang *et al* (1979). A mass spectrometer was used in conjunction with the computer control system to monitor the off gases from the fermentation and to control the addition of feed to maintain a set growth rate (eg 0.1 h^{-1}). Maximum conversion of carbon substrate into biomass is achieved by maintaining the respiratory coefficient below 1.2 (Collins, 1990) and, by this means, cell densities of approximately 100 g/L cell dry weight can be achieved. The culture supernatants were compared with those of a wild-type rHA producer by Coomassie-stained SDS/PAGE and by Western blot. These indicated (Figure 2) that, whilst elimination of the monobasic Arg^{410} (pAYE464) did reduce the level of the fragment by a useful amount, removal of both potential protease sites (pAYE468) almost abolished the 45kDa fragment.

The above data suggested that the generation of rHA fragment might be due to endoproteolytic attack, though the absence of an effect of removal of the potential Kex2p site $Lys^{413}Lys^{414}$ (Sleep *et al*, 1990, and confirmed by other studies not noted here) unless combined with elimination of Arg^{410}, had suggested a complex etiology. The reduction in the amount of fragment with the mutated rHA could in principle be due to an effect of the changes on the kinetics of folding of the molecule and not due to the removal of protease cleavage sites.

Example 2: Disruption of the *YAP3* gene.

The *YAP3* gene encoding yeast aspartyl protease 3 was mutated by the process of gene disruption (Rothstein 1983) which effectively deleted part of the *YAP3* coding sequence, thereby preventing the production of active Yap3p.

Four oligonucleotides suitable for PCR amplification of the 5′ and 3′ ends of the *YAP3* gene (Egel-Mitani *et al*, 1990) were synthesised using an Applied

Biosystems 380B Oligonucleotide Synthesiser. To assist the reader, we include as SEQ15 the sequence of the *YAP3* gene, of which 541-2250 is the coding sequence.

5′ end

YAP3A: 5′-CGTCAGACCTTGCATGCAGCCAAGACACCCTCACATAGC-3′ (SEQ5)

YAP3B: 5′-CCGTTACGTTCTGTGGTGGCATGCCCACTTCCAAGTCCACCG-3′ (SEQ6)

3' end

YAP3C: 5′-GCGTCTCATAGTGGAAAAGCTTCTAAATACGACAACTTCCCC-3′ (SEQ7)

YAP3D: 5′-CCCAAAATGGTACCTGTGTCATCACTCGTTGGGATAATACC-3′ (SEQ8)

PCR reactions were carried out to amplify individually the 5′ and 3′ ends of the *YAP3* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories, Inc). Conditions were as follows: 2.5μg/ml genomic DNA, 5μg/ml of each primer, denature at 94°C 61 seconds, anneal at 37°C 121 secs, extend at 72°C 181 secs for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size. The 5′ fragment was digested with *Sph*I and cloned into the *Sph*I site of pUC19HX (pUC19 lacking a *Hin*dIII site) to give pAYE511 (Figure 3), in which the orientation is such that *YAP3* would be transcribed towards the *Kpn*I site of the pUC19HX polylinker. The 3′ *YAP3* fragment was digested with *Hin*dIII and *Asp*718 (an isoschizomer of *Kpn*I) and ligated into pUC19 digested with *Hin*dIII/*Asp*718 to give pAYE512. Plasmid DNA sequencing was carried out on the inserts to confirm that the desired sequences had been cloned. The *Hin*dIII/*Asp*718 fragment of pAYE512 was then subcloned into the corresponding sites of pAYE511 to give pAYE513 (Fig 3), in which the 5′ and 3′ regions of *YAP3*

are correctly orientated with a unique *Hin*dIII site between them. The *URA3* gene was isolated from YEp24 (Botstein *et al*, 1979) as a *Hin*dIII fragment and then inserted into this site to give pAYE515 (Fig 3), with *URA3* flanked by the 5′ and 3′ regions of *YAP3*, and transcribed in the opposite direction to *YAP3*.

A *ura3* derivative of strain DB1 cir° pAYE316 (Sleep *et al*, 1991) was obtained by random chemical mutagenesis and selection for resistance to 5-fluoro-orotic acid (Boeke *et al*, 1987). The strain was grown overnight in 100 mL buffered minimal medium and the cells were collected by centrifugation and then washed once with sterile water. The cells were then resuspended in 10 mL sterile water and 2 mL aliquots were placed in separate 15 mL Falcon tubes. A 5 mg/mL solution of N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was then added to the tubes as follows: 0 μL, 20 μL, 40 μL, 80 μL or 160 μL. The cells were then incubated at 30°C for 30 min and then centrifuged and washed three times with sterile water. Finally, the cells were resuspended in 1 mL YEP (1% w/v yeast extract, 2% w/v Bacto peptone) and stored at 4°C. The percentage of cells that survived the mutagenic treatment was determined by spreading dilutions of the samples on YEP plates containing 2% w/v sucrose and incubating at 30°C for 3 days. Cells from the treatment which gave approximately 50% survival were grown on YEP plates containing 2% w/v sucrose and then replica-plated onto YNB minimal medium containing 2% w/v sucrose and supplemented with 5-fluoro-orotic acid (1 mg/mL) and uracil (50 μg/mL). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation and that this defect could be corrected by introduction of the *URA3* gene by transformation. One such strain, DBU3 cir° pAYE316, was transformed with the *Sph*I/*Asp*718 *YAP3-URA3-YAP*3 fragment of pAYE515 with selection for Ura^+ colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5′ and 3′ ends of the *YAP3* gene and confirmed the disruption of the *YAP3* gene. An anti-HSA Western blot of

YEPS shake-flask supernatants of two transformants indicated that disruption of *YAP3* markedly reduced rHA fragment levels.

One *yap3* derivative of DBU3 cir° pAYE316, designated DXY10 cir° pAYE316, was grown several times by fed-batch fermentation in minimal medium to high cell dry weight. When supernatants were examined by Coomassie-stained PAGE and anti-HSA Western blot (Figs 4 and 5), the reduction in the level of rHA 45kDa fragment was clearly apparent; estimates of the amount of the degradation product vary from $^1/_3$ to $^1/_5$ of the levels seen with the *YAP3* parent. The amount of rHA produced was not adversely affected by the *yap3* mutation, indeed DXY10 cir° pAYE316 was found to produce 30-50% more rHA than the *YAP3* equivalent, DB1 cir° pAYE316. Despite the fact that cleavage of the leader sequence from the HA sequence is C-terminal to a pair of basic residues, the rHA was found to have the correct N-terminus.

The fermentation broth was centrifuged to remove the cells and then subject to affinity chromatographic purification as follows. The culture supernatant was passed through a Cibacron Blue F3GA Sepharose column (Pharmacia) which was then washed with 0.1M phosphate glycine buffer, pH8.0. The rHA was then eluted from the column with 2M NaCl, 0.1M phosphate glycine, pH8.0, at which point it was >95% pure. It may be purified further by techniques known in the art.

The albumin may alternatively be purified from the culture medium by any of the variety of known techniques for purifying albumin from serum or fermentation culture medium, for example those disclosed in WO 92/04367, Maurel *et al* (1989), Curling (1980) and EP 524 681.

Example 3: Disruption of the *KEX2* gene in a *yap3* strain.

To construct a strain lacking both Yap3p and Kex2p activity, a *lys2* derivative of yeast strain DXY10 cir° (pAYE316) was obtained by random chemical mutagenesis and selection for resistance to α-amino adipate (Barnes and Thorner, 1985). Cells were mutagenised as in Example 2 and then plated on YNB minimal medium containing 2% w/v sucrose and supplemented with 2 mg/mL DL-α-amino adipate as the sole nitrogen source and 30 μg/mL lysine. Colonies able to grow on this medium were purified and tested to verify that they were unable to grow in the absence of lysine supplementation and that this defect could be corrected by the introduction of the *LYS2* gene by transformation. This strain was then mutated by the process of gene disruption which effectively disrupted part of the *KEX2* coding sequence, thereby preventing production of active Kex2p. To assist the reader, the sequence of the *KEX2* gene is reproduced herein as SEQ14, of which 1329-3773 is the coding sequence.

Four oligonucleotides suitable for PCR amplification of the 5′ and 3′ ends of the *KEX2* gene (Fuller *et al*, 1989) were synthesised using an Applied Biosystems 380B Oligonucleotide Synthesiser.

5′ end

KEX2A: 5′-CCATCTGGATCCAATGGTGCTTTGGCCAAATAAATAGTTTCAGC-3′ (SEQ9)

KEX2B: 5′-GCTTCTTTTACCGGTAACAAGCTTGAGTCCATTGG-3′ (SEQ10)

3′ end

KEX2C: 5′-GGTAAGGTTTAGTCGACCTATTTTTTGTTTTGTCTGC-3′ (SEQ11)

KEX2D: 5′-GGAAACGTATGAATTCGATATCATTGATACAGACTCTGAGTACG-3′ (SEQ12)

PCR reactions were carried out to amplify individually the 5′ and 3′ ends of the *KEX2* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories Inc). Conditions were as follows: 2.5 μg/ml genomic DNA, 5 μg/ml of each primer, denature 94°C 61s, anneal 37°C 121s, extend 72°C 181s for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size (0.9 kb for the 5′ product and 0.62 kb for the 3′ product). The 5′ product was digested with *Bam*HI and *Hin*dIII and the 3′ product was digested with *Hin*dIII and *Sal*I and then the two fragments were together cloned into pUC19HX digested with *Bam*HI and *Sal*I. A 4.8 kb *Hin*dIII fragment comprising the *S. cerevisiae LYS2* gene (Barnes & Thorner, 1985) was then inserted into the resulting plasmid at *Hin*dIII (ie between the two *KEX2* fragments) to form pAYE519 (Fig 6).

The *lys2* derivative of DXY10 cir° (pAYE316), *lys2-16*, was transformed with the 6.0 kb *KEX2-LYS2-KEX2* fragment of pAYE519, selecting for Lys^+ colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5′ and 3′ ends of the *KEX2* gene and confirmed the disruption of the *KEX2* gene. An anti-HSA Western blot of YEPS shake-flask culture supernatants of these transformants indicated that disruption of *KEX2* in a *yap3* strain reduced the level of rHA fragment still further, despite the lack of an effect of disruption of *KEX2* alone in Example 4 below. Analysis of the rHA produced by one such strain, ABB50, indicated that the leader sequence was incorrectly processed, leading to an abnormal N-terminus.

The strain ABB50 (pAYE316) was cured of its plasmid (Sleep *et al*, 1991) and transformed with a similar plasmid, pAYE522, in which the hybrid leader sequence was replaced by the *S. cerevisiae* invertase (*SUC2*) leader sequence

such that the encoded leader and the junction with the HSA sequence were as follows:

MLLQAFLFLLAGFAAKISA↓DAHKS (SEQ13)

Invertase leader HSA

In this construct, cleavage of the leader sequence from HSA does not rely upon activity of the Kex2 protease. The strain ABB50 (pAYE522) was found to produce rHA with a similarly very low level of rHA fragment, but in this instance the N-terminus corresponded to that of serum-derived HSA, ie there was efficient and precise removal of the leader sequence.

Example 4: Disruption of the *KEX2* gene alone (Comparative Example).

By a similar method to that disclosed in Example 3 the *KEX2* gene was disrupted in *S. cerevisiae*. This strain had the Yap3p proteolytic activity and was therefore not within the scope of the invention. When this strain was grown in fed batch fermentation the rHA produced contained similar amounts of fragment to that produced by strains with an intact *KEX2* gene. In addition, the overall level of rHA was reduced and the leader sequence was not correctly processed, leading to an abnormal N-terminus.

Example 5: Identification of equivalent protease in *Pichia*.

As noted above, non-*Saccharomyces* yeast similarly produce the undesirable fragment of rHA and therefore have the Yap3p proteolytic activity. We have confirmed this by performing Southern hybridisations of *Pichia angusta* DNA, using the *S. cerevisiae YAP3* gene as a probe. A specific DNA fragment was identified, showing that, not only is the Yap3p proteolytic activity present in *P. angusta*, but a specific homologue of the *YAP3* gene is present also.

The method of Southern hybridization used for detection of the *YAP3* homologue can be adapted to clone the gene sequence from a genomic DNA library of *Pichia* DNA using standard procedures (Sambrook *et al*, 1989). Disruption of the *YAP3* homologue in *Pichia sp.* can be achieved using similar techniques to those used above for *Saccharomyces* (Cregg and Madden, 1987).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(C) CITY: Nottingham
(D) STATE: Nottinghamshire
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): NG7 1FD

(ii) TITLE OF INVENTION: Yeast strains and modified albumins

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1830 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 73..1827

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Cys Ala Asp Asp Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln
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TTC CAT GCA GAT ATA TGC ACA CTT TCT GAG AAG GAG AGA CAA ATC AAG      1644
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525                 530                 535                 540

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            560                 565                 570

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 585 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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            100                 105                 110

Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
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    130                 135                 140

Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
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Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
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225                 230                 235                 240

Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
                245                 250                 255

Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
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Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
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Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser
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290 295 300

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325 330 335

Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr
340 345 350

Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu
355 360 365

Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro
370 375 380

Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
385 390 395 400

Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro
405 410 415

Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys
420 425 430

Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys
435 440 445

Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His
450 455 460

Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser
465 470 475 480

Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr
485 490 495

Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp
500 505 510

Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala
515 520 525

Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu
530 535 540

Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys
545 550 555 560

Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
565 570 575

Ala Ala Ser Gln Ala Ala Leu Gly Leu
580 585

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Tyr Thr Lys Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Tyr Thr Gln Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGTCAGACCT TGCATGCAGC CAAGACACCC TCACATAGC 39

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCGTTACGTT CTGTGGTGGC ATGCCCACTT CCAAGTCCAC CG 42

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCGTCTCATA GTGGAAAAGC TTCTAAATAC GACAACTTCC CC 42

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCCAAAATGG TACCTGTGTC ATCACTCGTT GGGATAATAC C 41

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCATCTGGAT CCAATGGTGC TTTGGCCAAA TAAATAGTTT CAGC 44

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCTTCTTTTA CCGGTAACAA GCTTGAGTCC ATTGG 35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGTAAGGTTT AGTCGACCTA TTTTTTGTTT TGTCTGC 37

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGAAACGTAT GAATTCGATA TCATTGATAC AGACTCTGAG TACG 44

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Met Leu Leu Gln Ala Phe Leu Phe Leu Leu Ala Gly Phe Ala Ala Lys
1 5 10 15

Ile Ser Ala Asp Ala His Lys Ser
20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4106 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GAATTCTCTG TTGACTACTA AACTGAGAGA ATTTGCCGAG ACTCTAAGAA CAGCTTTGAA 60

AGAGCGTTCT GCCGATGATT CCATAATTGT CACTCTGAGA GAGCAAATGC AAAGAGAAAT 120

CTTCAGGTTG ATGTCGTTGT TCATGGACAT ACCTCCAGTG CAACCAAACG AGCAATTCAC 180

TTGGGAATAC GTTGACAAAG ACAAGAAAAT CCACACTATC AAATCGACTC CGTTAGAATT 240

TGCCTCCAAA TACGCAAAAT TGGACCCTTC CACGCCAGTC TCATTGATCA ATGATCCAAG 300

ACACCATATG GTAAATTAAT TAAGATCGAT CGTTTAGGAA ACGTCCTTGG CGGAGATGCC 360

GTGATTTACT TAAATGTTGA CAATGAAACA CTATCTAAAT TGGTTGTTAA GAGATTACAA 420

AATAACAAAG CTGTCTTTTT TGGATCTCAC ACTCCAAAGT TCATGGACAA GAAAACTGGT 480

GTCATGGATA TTGAATTGTG GAACTATCCT GCCATGGCTA TAATTTACCT CAGCAAAAGG 540

CATCCGGTAT TAGATACCAT GAAAGTTTGA TGACTCATGC TATGTTGGAT CACTGGCTGC 600

CACGTCGATG AAACGTCTAA ATTACCACTT CGCTACCGTC TGAAAATTCC TGGGGTAAAG 660

ACTCCGGTAA AGACGGATTA TACGTGATGA CTCAAAAGTA CTTCGAGGAG TACTGCTTTC 720

AAATTGTGGT CGATATCAAT GAATTGCCAA AAGAGCTGGC TTCAAAATTC ACCTCAGGTA 780

AGGAAGAGCC GATTGTCTTG CCCATCTGGA CCCAATGGTG CTTTGGCCAA ATAAATAGTT 840

TCAGCAGCTC TGATGTAGAT ACACGTATCT CGACATGTTT TATTTTTACT ATACATACAT 900

AAAAGAAATA AAAAATGATA ACGTGTATAT TATTATTCAT ATAATCAATG AGGGTCATTT 960

TCTGAAACGC AAAAAACGGT AAATGGAAAA AAAATAAAGA TAGAAAAAGA AAACAAACAA 1020

AGGAAAGGTT AGCATATTAA ATAACTGAGC TGATACTTCA ACAGCATCGC TGAAGAGAAC 1080

AGTATTGAAA CCGAAACATT TTCTAAAGGC AAACAAGGTA CTCCATATTT GCTGGACGTG 1140

TTCTTTCTCT CGTTTCATAT GCATAATTCT GTCATAAGCC TGTTCTTTTT CCTGGCTTAA 1200

ACATCCCGTT TTGTAAAAGA GAAATCTATT CCACATATTT CATTCATTCG GCTACCATAC 1260

TAAGGATAAA CTAATCCCGT TGTTTTTTGG CCTCGTCACA TAATTATAAA CTACTAACCC 1320

ATTATCAGAT GAAAGTGAGG AAATATATTA CTTTATGCTT TTGGTGGGCC TTTTCAACAT 1380

CCGCTCTTGT ATCATCACAA CAAATTCCAT TGAAGGACCA TACGTCACGA CAGTATTTTG 1440

CTGTAGAAAG CAATGAAACA TTATCCCGCT TGGAGGAAAT GCATCCAAAT TGGAAATATG 1500

AACATGATGT TCGAGGGCTA CCAAACCATT ATGTTTTTTC AAAAGAGTTG CTAAAATTGG 1560

GCAAAAGATC ATCATTAGAA GAGTTACAGG GGGATAACAA CGACCACATA TTATCTGTCC 1620

ATGATTTATT CCCGCGTAAC GACCTATTTA AGAGACTACC GGTGCCTGCT CCACCAATGG 1680

ACTCAAGCTT GTTACCGGTA AAAGAAGCTG AGGATAAACT CAGCATAAAT GATCCGCTTT 1740

TTGAGAGGCA GTGGCACTTG GTCAATCCAA GTTTTCCTGG CAGTGATATA AATGTTCTTG 1800

ATCTGTGGTA CAATAATATT ACAGGCGCAG GGGTCGTGGC TGCCATTGTT GATGATGGCC 1860

TTGACTACGA AAATGAAGAC TTGAAGGATA ATTTTTGCGC TGAAGGTTCT TGGGATTTCA 1920

ACGACAATAC CAATTTACCT AAACCAAGAT TATCTGATGA CTACCATGGT ACGAGATGTG 1980

CAGGTGAAAT AGCTGCCAAA AAAGGTAACA ATTTTTGCGG TGTCGGGGTA GGTTACAACG 2040

CTAAAATCTC AGGCATAAGA ATCTTATCCG GTGATATCAC TACGGAAGAT GAAGCTGCGT 2100

CCTTGATTTA TGGTCTAGAC GTAAACGATA TATATTCATG CTCATGGGGT CCCGCTGATG 2160

ACGGAAGACA TTTACAAGGC CCTAGTGACC TGGTGAAAAA GGCTTTAGTA AAAGGTGTTA 2220

CTGAGGGAAG AGATTCCAAA GGAGCGATTT ACGTTTTTGC CAGTGGAAAT GGTGGAACTC 2280

GTGGTGATAA TTGCAATTAC GACGGCTATA CTAATTCCAT ATATTCTATT ACTATTGGGG 2340

CTATTGATCA CAAAGATCTA CATCCTCCTT ATTCCGAAGG TTGTTCCGCC GTCATGGCAG 2400

TCACGTATTC TTCAGGTTCA GGCGAATATA TTCATTCGAG TGATATCAAC GGCAGATGCA 2460

GTAATAGCCA CGGTGGAACG TCTGCGGCTG CTCCATTAGC TGCCGGTGTT TACACTTTGT 2520

TACTAGAAGC CAACCCAAAC CTAACTTGGA GAGACGTACA GTATTTATCA ATCTTGTCTG 2580

CGGTAGGGTT AGAAAAGAAC GCTGACGGAG ATTGGAGAGA TAGCGCCATG GGGAAGAAAT 2640

ACTCTCATCG CTATGGCTTT GGTAAAATCG ATGCCCATAA GTTAATTGAA ATGTCCAAGA 2700

CCTGGGAGAA TGTTAACGCA CAAACCTGGT TTTACCTGCC AACATTGTAT GTTTCCCAGT 2760

CCACAAACTC CACGGAAGAG ACATTAGAAT CCGTCATAAC CATATCAGAA AAAAGTCTTC 2820

AAGATGCTAA CTTCAAGAGA ATTGAGCACG TCACGGTAAC TGTAGATATT GATACAGAAA 2880

TTAGGGGAAC TACGACTGTC GATTTAATAT CACCAGCGGG GATAATTTCA AACCTTGGCG 2940

TTGTAAGACC AAGAGATGTT TCATCAGAGG GATTCAAAGA CTGGACATTC ATGTCTGTAG 3000

CACATTGGGG TGAGAACGGC GTAGGTGATT GGAAAATCAA GGTTAAGACA ACAGAAAATG 3060

GACACAGGAT TGACTTCCAC AGTTGGAGGC TGAAGCTCTT TGGGGAATCC ATTGATTCAT 3120

CTAAAACAGA AACTTTCGTC TTTGGAAACG ATAAAGAGGA GGTTGAACCA GCTGCTACAG 3180

AAAGTACCGT ATCACAATAT TCTGCCAGTT CAACTTCTAT TTCCATCAGC GCTACTTCTA 3240

CATCTTCTAT CTCAATTGGT GTGGAAACGT CGGCCATTCC CCAAACGACT ACTGCGAGTA 3300

CCGATCCTGA TTCTGATCCA AACACTCCTA AAAAACTTTC CTCTCCTAGG CAAGCCATGC 3360

ATTATTTTTT AACAATATTT TTGATTGGCG CCACATTTTT GGTGTTATAC TTCATGTTTT 3420

TTATGAAATC AAGGAGAAGG ATCAGAAGGT CAAGAGCGGA AACGTATGAA TTCGATATCA 3480

TTGATACAGA CTCTGAGTAC GATTCTACTT TGGACAATGG AACTTCCGGA ATTACTGAGC 3540

CCGAAGAGGT TGAGGACTTC GATTTTGATT TGTCCGATGA AGACCATCTT GCAAGTTTGT 3600

CTTCATCAGA AAACGGTGAT GCTGAACATA CAATTGATAG TGTACTAACA AACGAAAATC 3660

CATTTAGTGA CCCTATAAAG CAAAAGTTCC CAAATGACGC CAACGCAGAA TCTGCTTCCA 3720

ATAAATTACA AGAATTACAG CCTGATGTTC CTCCATCTTC CGGACGATCG TGATTCGATA 3780

TGTACAGAAA GCTTCAAATT ACAAAATAGC ATTTTTTTCT TATAGATTAT AATACTCTCT 3840

CATACGTATA CGTATATGTG TATATGATAT ATAAACAAAC ATTAATATCC TATTCCTTCC 3900

GTTTGAAATC CCTATGATGT ACTTTGCATT GTTTGCACCC GCGAATAAAA TGAAAACTCC 3960

GAACCGATAT ATCAAGCACA TAAAAGGGGA GGGTCCAATT AATGCATATT TAAGACCACA 4020

GCTGAATAAC TTTAAAACGG CAGACAAAAC AAAAAATAGG TCGAATAAAC CTTACCTGCC 4080

TAGAAGGAAT GACAGCAGCT AATAAG 4106

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2526 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Saccharomyces cerevisiae

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCGTTTTCTT TTCGTAAAAA AAAACAATAG ACACTATATA TAGACACTTT TTCCTTTCCT 60

TCTTTGCGCG ATTTCAAGAG GAAAAGCATA CTTAAATAAG AATATTCCTA AAACACACGT 120

TCTGACGCGT CAATTAGATC GTCAGACCTT GCATGCAGCC AAGACACCCT CACATAGCAC 180

TGCCTCCTTC CTCCTCTTTT CTGTCACCAC CTCACCTCCC TCGTCCACTC AACTGAGTGG 240

CTTTTCGCTC CTTTTATACT GCGCCATGAG TAGTTTTCGT TTCACTGATG TGTCCGAAAA 300

AATTGAGGTT TCATAAAAAA ATTCGTGGAC TTATTTATGG AGAAACAGGG AAATCCGACT 360

ACTTAAGAAA AGGGTGTCAA AGAGGATTTA CTTTTTTCCT TCTTTTTGCA TTTGTTCCTA 420

TTTCCGCAAT TGGACGGTTA TTAAGAAGAA CGCAATTGGC TTTTCTGTAT ATTAAAATAC 480

ATAGCGTAAT AAAAAGATAA GGTGAACACC AAGCATATAG TATAATATTA CCTACCACAT 540

ATGAAACTGA AAACTGTAAG ATCTGCGGTC CTTTCGTCAC TCTTTGCATC GCAGGTTCTC 600

GGTAAGATAA TACCAGCAGC AAACAAGCGC GACGACGACT CGAATTCCAA GTTCGTCAAG 660

TTGCCCTTTC ATAAGCTTTA CGGGGACTCG CTAGAAAATG TGGGAAGCGA CAAAAAACCG 720

GAAGTACGCC TATTGAAGAG GGCTGACGGT TATGAAGAAA TTATAATTAC CAACCAGCAA 780

AGTTTCTATT CGGTGGACTT GGAAGTGGGC ACGCCACCAC AGAACGTAAC GGTCCTGGTG 840

GACACAGGCT CCTCTGATCT ATGGATTATG GGCTCGGATA ATCCATACTG TTCTTCGAAC 900

AGTATGGGTA GTAGCCGGAG ACGTGTTATT GACAAACGTG ATGATTCGTC AAGCGGCGGA 960

TCTTTGATTA ATGATATAAA CCCATTTGGC TGGTTGACGG GAACGGGCAG TGCCATTGGC 1020

CCCACTGCTA CGGGCTTAGG AGGCGGTTCA GGTACGGCAA CTCAATCCGT GCCTGCTTCG 1080

GAAGCCACCA TGGACTGTCA ACAATACGGG ACATTTTCCA CTTCGGGCTC TTCTACATTT 1140

AGATCAAACA ACACCTATTT CAGTATTAGC TACGGTGATG GGACTTTTGC CTCCGGTACT 1200

TTTGGTACGG ATGTTTTGGA TTTAAGCGAC TTGAACGTTA CCGGGTTGTC TTTTGCCGTT 1260

GCCAATGAAA CGAATTCTAC TATGGGTGTG TTAGGTATTG GTTTGCCCGA ATTAGAAGTC 1320

ACTTATTCTG GCTCTACTGC GTCTCATAGT GGAAAAGCTT ATAAATACGA CAACTTCCCC 1380

ATTGTATTGA AAAATTCTGG TGCTATCAAA AGCAACACAT ATTCTTTGTA TTTGAACGAC 1440

TCGGACGCTA TGCATGGCAC CATTTTGTTC GGAGCCGTGG ACCACAGTAA ATATACCGGC 1500

ACCTTATACA CAATCCCCAT CGTAAACACT CTGAGTGCTA GTGGATTTAG CTCTCCCATT 1560

CAATTTGATG TCACTATTAA TGGTATCGGT ATTAGTGATT CTGGGAGTAG TAACAAGACC 1620

TTGACTACCA CTAAAATACC TGCTTTGTCG GATTCCGGTA CTACTTTGAC TTATTTACCT 1680

CAAACAGTGG TAAGTATGAT CGCTACTGAA CTAGGTGCGC AATACTCTTC CAGGATAGGG 1740

TATTACGTAT TGGACTGTCC ATCTGATGAT AGTATGGAAA TAGTGTTCGA TTTTGGTGGT 1800

TTTCACATCA ATGCACCACT TTCGAGTTTT ATCTTGAGTA CTGGCACTAC ATGTCTTTTA 1860

GGTATTATCC CAACGAGTGA TGACACAGGT ACCATTTTGG GTGATTCATT TTTGACTAAC 1920

GCGTACGTGG TTTATGATTT GGAGAATCTT GAAATATCCA TGGCACAAGC TCGCTATAAT 1980

ACCACAAGCG AAAATATCGA AATTATCACA TCCTCTGTTC CAAGCGCCGT AAAGGCACCA 2040

GGCTATACAA ACACTTGGTC CACAAGTGCA TCTATTGTTA CCGGTGGTAA CATATTTACT 2100

GTAAATTCCT CACAAACTGC TTCCTTTAGC GGTAACCTGA CGACCAGTAC TGCATCCGCC 2160

ACTTCTACAT CAAGTAAAAG AAATGTTGGT GATCATATAG TTCCATCTTT ACCCCTCACA 2220

TTAATTTCTC TTCTTTTTGC ATTCATCTGA AAACCGTTGC ACAAAGTTTA GACATTCACA 2280

TCTCCAAGCC AGTTGGAGTT TCTGGCGGAA ATCGTTGCTC TCGCTTGGGC AAAGTTTTTT 2340

TTTATTATTA ATTTTTTATT GTTACGTTGG CGGTCTTTAT TTTTACTTCA CAATAGTTTA 2400

TCTTACCCAC TAAGAATAGG TTACCATTTA TTCACATTTT TTTTTCTCAT TCCTAGTATA 2460

CTATTTACCT GGGATATGGC CTATAATCAA AGGCTTTAAT ATTCTAATAA TTCGTTTGGC 2520

ATCTAG 2526

CLAIMS

1. A process for preparing albumin by secretion from a yeast genetically modified to produce and secrete the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.

2. A process according to Claim 1 wherein the said proteolytic activity is an endoprotease activity specific for monobasic sites and for paired basic amino acids in a polypeptide.

3. A process according to Claim 1 or 2 wherein the yeast is *S. cerevisiae*.

4. A process according to Claim 1, 2 or 3 wherein the yeast lacks a functional *YAP3* gene or homologue thereof.

5. A process according to any one of Claims 1 to 4 wherein the yeast cells additionally have a reduced level of *S. cerevisiae* Kex2p proteolytic activity.

6. A process according to any one of the preceding claims wherein the albumin is a human albumin.

7. A culture of yeast cells containing a polynucleotide sequence encoding an albumin and a second polynucleotide sequence encoding a secretion signal causing albumin expressed from the first polynucleotide sequence to be secreted from the yeast, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic

activity.

8. A culture according to Claim 7 wherein the albumin is a human albumin.

9. A culture according to Claim 7 or 8 wherein the yeast is *S. cerevisiae*.

10. A culture according to any one of Claims 7 to 9 wherein the said signal is cleaved by the yeast prior to release of the albumin from the yeast.

11. A culture according to any one of Claims 7 to 10 wherein the yeast cells additionally have a reduced level of Kex2p proteolytic activity.

12. A culture according to Claim 11 wherein the said secretion signal is cleaved from the albumin by a protease other than Kex2p.

13. A modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with yeast aspartyl protease 3 (Yap3p) when expressed and secreted in yeast, characterised in that the modified albumin is not susceptible to such cleavage.

14. A modified albumin according to Claim 13 wherein the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein.

15. A modified albumin according to Claim 13 or 14 wherein the said monobasic amino acid is arginine.

16. A modified albumin according to Claim 14 or 15 wherein the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin.

17. A modified albumin according to Claim 16 wherein the said pair of amino acids is Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg.

18. A modified albumin according to Claim 13 wherein the naturally-occurring albumin is a human albumin and the modified protein lacks Arg^{410}; and, optionally, residues 413 and 414 do not each consist of lysine or arginine.

19. A modified albumin according to Claim 18 which is a human albumin having the amino acid changes R410A, K413Q, K414Q.

20. A polynucleotide encoding a modified albumin according to any one of Claims 13 to 19.

21. A yeast containing a polynucleotide according to Claim 20, transcription signals such that the modified albumin is expressed in the yeast, and a further polynucleotide adjacent the said polynucleotide such that the modified albumin is secreted from the yeast.

Figure 1

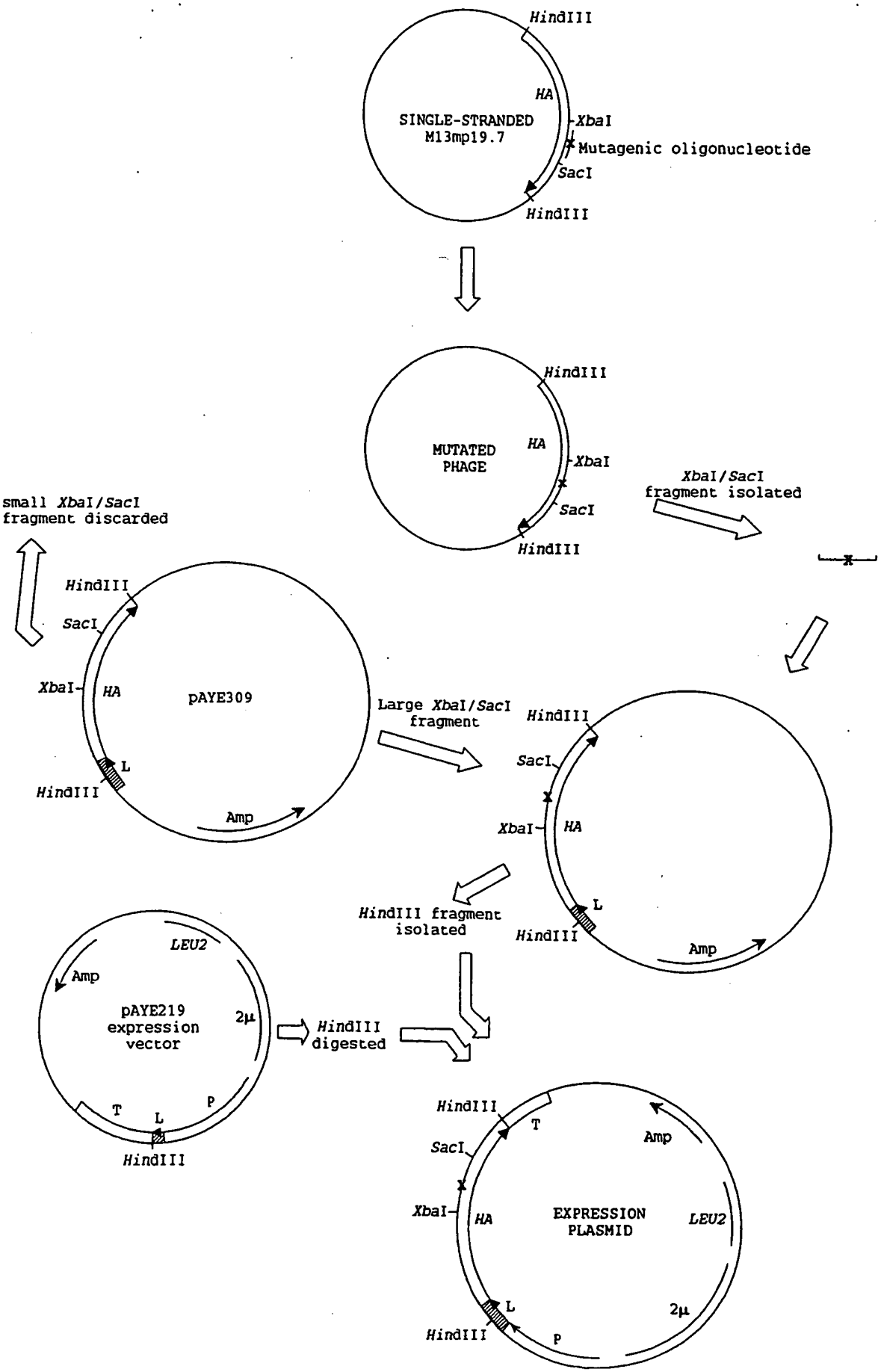

HindIII
HA
SINGLE-STRANDED
M13mp19.7
XbaI
Mutagenic oligonucleotide
SacI
HindIII
HindIII
MUTATED
PHAGE
HA
XbaI
SacI
HindIII
XbaI/SacI
fragment isolated
small XbaI/SacI
fragment discarded
HindIII
SacI
XbaI
HA
pAYE309
L
HindIII
Amp
Large XbaI/SacI
fragment
HindIII
SacI
XbaI
HA
L
HindIII
Amp
HindIII fragment
isolated
LEU2
Amp
pAYE219
expression
vector
2μ
HindIII
digested
T
L
P
HindIII
HindIII
T
Amp
SacI
XbaI
HA
EXPRESSION
PLASMID
LEU2
L
2μ
HindIII
P

Figure 2

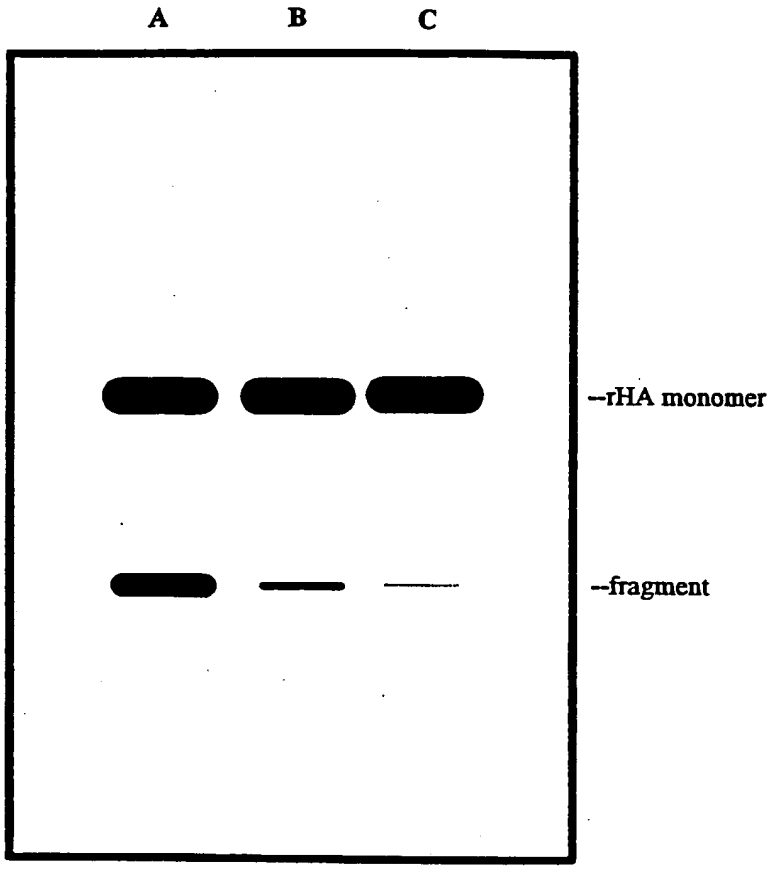


Figure 3

5' and 3' regions of *YAP3* obtained by PCR:

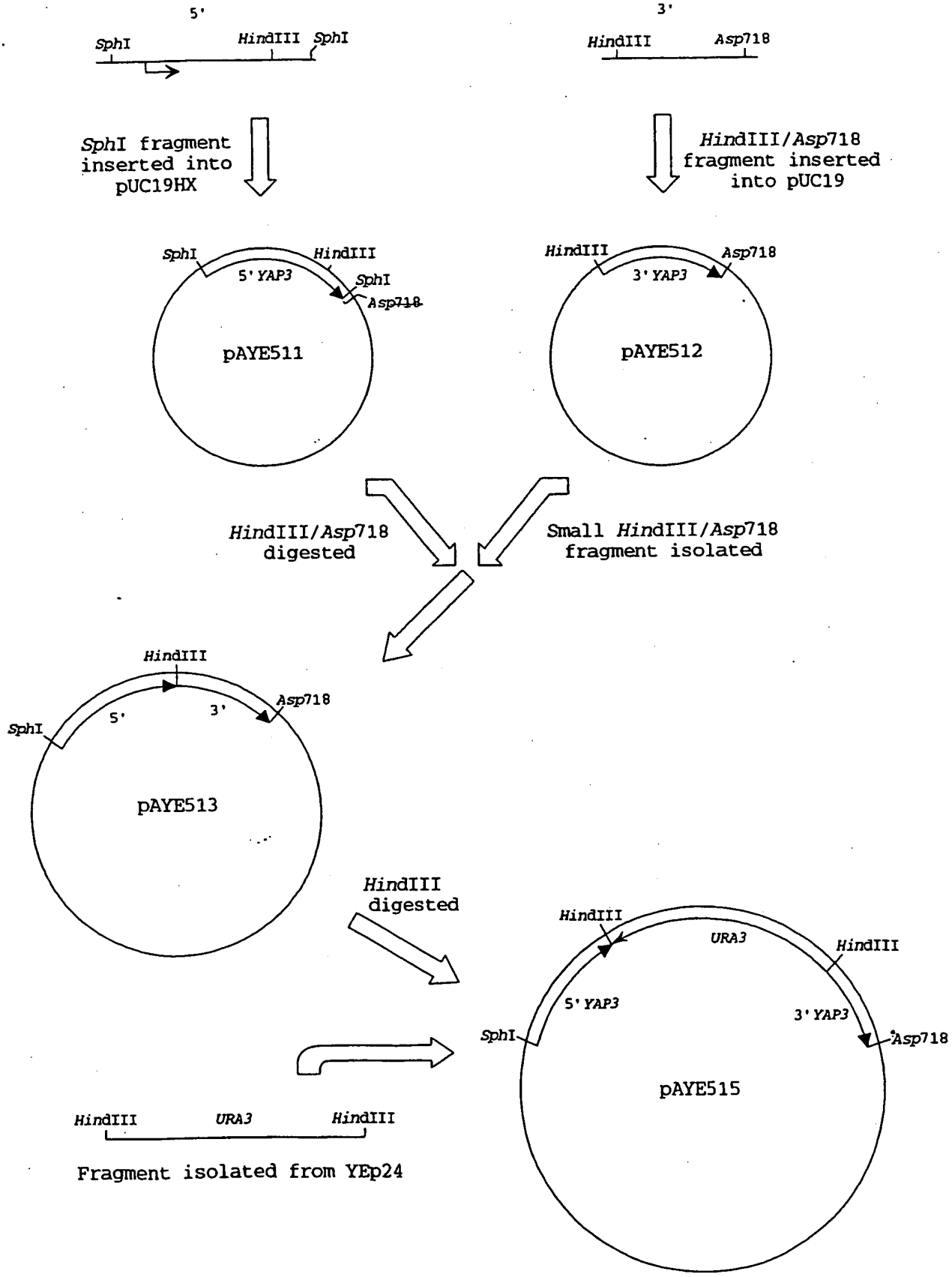


Figure 4

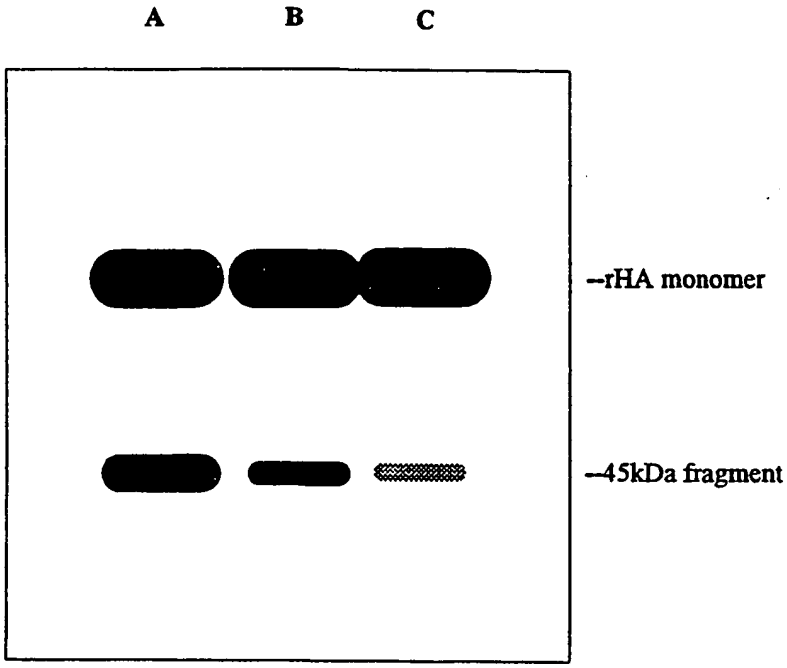


Figure 5

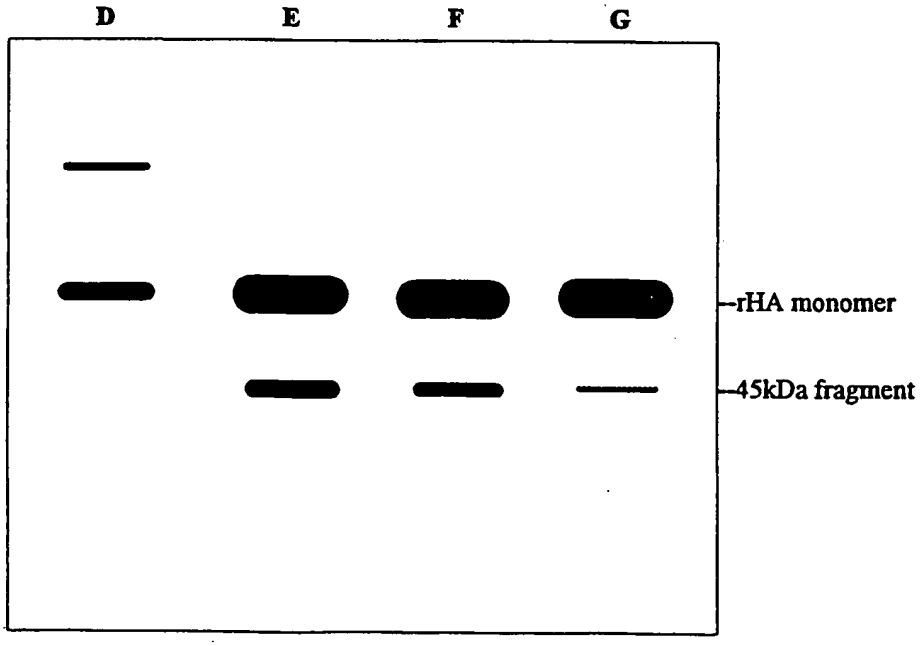
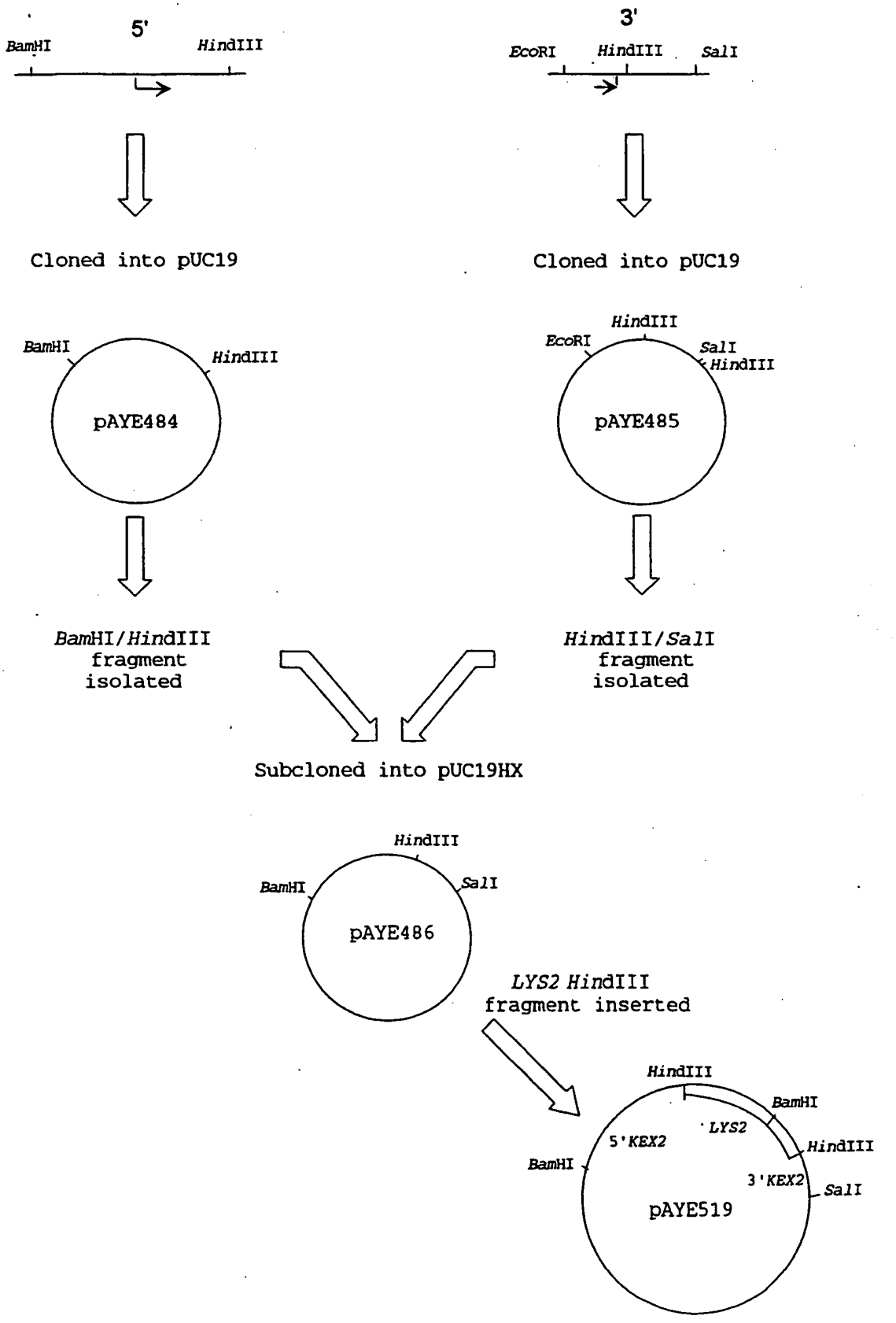


Figure 6


5' and 3' regions of KEX2 obtained by PCR:
5'
BamHI
HindIII
3'
EcoRI
HindIII
SalI
Cloned into pUC19
Cloned into pUC19
BamHI
HindIII
pAYE484
HindIII
EcoRI
SalI
HindIII
pAYE485
BamHI/HindIII fragment isolated
HindIII/SalI fragment isolated
Subcloned into pUC19HX
HindIII
BamHI
SalI
pAYE486
LYS2 HindIII fragment inserted
HindIII
BamHI
LYS2
5'KEX2
HindIII
BamHI
3'KEX2
SalI
pAYE519

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N1/21 A61K38/38 C12P21/02 C12N15/11
C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	BIOCHIMIE, vol. 76, 1994 pages 226-233, Y. BOURBONNAIS ET AL. 'Cleavage of prosomatostatins by the yeast Yap3 and Kex2 endoprotease' *see the whole article* ---	1-21
Y	BIOTECHNOLOGY, vol. 8, no. 1, 1990 pages 42-46, D. SLEEP ET AL. 'The secretion of human serum albumin from the yeast S. cerevisiae using five different leader sequences' *see the whole article* ---	1-21

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
23 June 1995	-5. 07. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Marie, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO JOURNAL, vol. 12, no. 1, 1993 pages 285-294, Y. BOURBONNAIS ET AL. 'Isolation and characterisation of S. cerevisiae mutants defective in somatostatin expression...' *see the whole article* ---	1-21
Y	YEAST, vol. 6, 1990 pages 127-137, M. EGEL-MITANI ET AL. 'A novel aspartyl protease allowing KEX2-independent MF alfa propheromone processing in yeast' *see the whole article* -----	1-21